Expression of the Interleukin-3 and Granulocyte-Macrophage Colony-Stimulating Factor Genes in Friend Spleen Focus-Forming Virus-Induced Erythroleukemia

By Yoshihiro Shimada, Giovanni Migliaccio, Sandra Ruscetti, John W. Adamson, and Anna Rita Migliaccio

Friend spleen focus-forming virus (F-SFFV) is a replication-defective retrovirus that induces a multistage erythroleukemia in mice. In the first stage, expression of the SFFV envelope glycoprotein results in erythroid hyperplasia. Subsequently, the F-SFFV integrates near the Spi-1 gene and activates its expression, resulting in immortalized cells that represent a second stage in the disease process. We report here that media conditioned by erythroleukemia cell lines or leukemic spleen cells induced by the polycythemia-inducing strain of F-SFFV (F-SFFVp), but not medium conditioned by SFFVp-induced hyperplastic spleens, promote the proliferation of normal granulocyte-macrophage colony-stimulating factor progenitor cells and of granulocyte-macrophage colony-stimulating factor-stimulated factor (GM-CSF)– and/or interleukin-3 (IL-3)–dependent cell lines. The colony-stimulating activity of the conditioned media from four of five of the lines studied was neutralized by antibodies specific for IL-3 and/or GM-CSF, and IL-3 and GM-CSF–specific mRNA could be detected in the cells after amplification by the polymerase chain reaction. No rearrangements of the IL-3 or GM-CSF genes were observed by Southern blot analysis. However, as previously shown for SFFV-induced cell lines, the Spi-1 gene was expressed in all of these cells. Because the Spi-1 gene encodes a transcription factor whose cognate sequences are present in the promoter region of many hematopoietic growth factor genes, including IL-3 and GM-CSF, Spi-1 activation may be inducing the expression of these genes.

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Materials and Methods

Infection of BALB/c and DBA mice with the Friend SFFVp virus. DBA/2 and BALB/c mice (7- to 11-week-old males; Charles River Laboratories, Wilmington, MA) were injected intravenously (IV) with saturating doses of Friend virus (SFFVp + F-MuLV) prepared as described. Spenic enlargement was detectable by 2 to 3 weeks. The mice were then killed and single cell suspensions of their spleens were used to prepare conditioned medium (CM) or mRNA (see below). The spleens of mice injected with Iscove's Modified Dulbecco's Medium (IMDM) alone served as a source of control cells. A 20-fold increase in the weight of the spleen was observed.

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observed after infection (1.92 ± 0.75 vs. 0.09 ± 0.02 g/spleen in SFFVp- and mock-infected spleens, respectively). The capacity of the cells to grow as cell lines was established to distinguish between animals at the hyperplastic or leukemic stage of the disease.

Cell lines. MEL NP1, MEL NP5, MEL NP12, and MEL NP13 are erythroleukemia cell lines derived from mice infected with helper-free SFFVp. The DS19 erythroleukemia cell line was derived from a mouse infected with a Friend MuLV pseudotype of SFFVp. All cells were maintained by weekly passage in IMDM (GIBCO, Gaithersburg, MD) supplemented with antibiotics (100 U/mL of penicillin, 250 mg/mL of amphotericin B, and 100 µg/mL of streptomycin), β-mercaptoethanol (7.5 × 10⁻⁴ mol/L), L-glutamine, and fetal bovine serum (FBS) (5% vol/vol; HyClone, Logan, UT). The growth factor-dependent cell lines 32D cl 3 and FDC-P1 were passed twice in McCoy’s medium (GIBCO), modified as described by Greenberger et al., and supplemented with antibiotics, β-mercaptoethanol, L-glutamine, horse serum, and FBS (10% vol/vol of each; HyClone), and 10% (vol/vol) of media conditioned by WEHI-3 cells as a source of IL-3.

Growth factors and antibodies. Pure recombinant murine IL-3 and GM-CSF were provided by Dr J.-J. Mermod (Glaxo, Geneva, Switzerland). The growth factors were used at concentrations previously shown to have maximal activity on the growth of cell lines or normal hematopoietic progenitors. The rat antimurine IL-3 monoclonal antibody (MoAb) and the rabbit GM-CSF antiserum were provided by Drs. J. Abrams (DNAX, Palo Alto, CA) and J. Egie (Amgen, Thousand Oaks, CA), respectively. The anti-IL-3 antibody (1:40 dilution) completely neutralized the 3HTdR incorporation of 32D cells induced by IL-3 (1 U/mL) but did not affect GM-CSF–induced 3HTdR uptake by the same cells. The anti-GM-CSF antibody (1:500 dilution) partially (79%) neutralized 3HTdR incorporation of FDC-P1 cells induced by GM-CSF (2 U/mL) but not 3HTdR uptake induced by IL-3 (5 U/mL).

Preparation of CM from erythroleukemia cell lines and chromatographic analysis of the stimulatory activities. CM from erythroleukemia cell lines or from primary cultures of spleen cells from mice infected with Friend SFFVp was obtained by incubating the cells (5 × 10⁵ cells/mL) at 37°C in IMDM supplemented either with FBS (5% vol/vol) or with a serum-substituting mixture (FBS-free) CM composed of iron-saturated transferrin (final concentration 20 µg/mL) and insulin (final concentration 10 µg/mL). After 48 hours the cells were removed by centrifugation (400g for 20 minutes), and the CM filtered and concentrated 10- to 20-fold with Centricon filters (Amicon, Beverly, MA) before being tested for biologic activity.

For chromatographic analysis, 2 L of FBS-free CM of MEL NP12 cells were concentrated 70-fold using a Miniant Acrylic Ultrafiltration column (Millipore, Bedford, MA). The concentrate was loaded onto a MonoQ anion exchange column (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated in 20 mmol/L Tris-HCl buffer (pH 8.0) and operated with a FPLC system (Pharmacia). The sample was eluted from the column with a stepwise gradient of NaCl (up to 0.8 mol/L). The collected fractions, after appropriate concentration, were desalted and tested for their capacity to stimulate 3HTdR incorporation of 32D and FDC-P1 cells.

Detection of hematopoietic growth factors in CM from erythroleukemia cell lines. The presence of hematopoietic growth factors in CM from erythroleukemia cell lines was detected on the basis of the capacity of the CM to sustain colony formation by normal GM progenitors or by growth factor–dependent cell lines. For general purposes, the presence of hematopoietic growth factors was detected by the induction of 3HTdR incorporation in growth factor–dependent cell lines.

Colony-forming assays with normal progenitors, FDC-P1 and 32D cells, and erythroleukemia cell lines. Assays of colony formation by normal cells (5 × 10⁴ cells/mL) or cell lines (5 × 10⁴ to 10⁵ cells/mL) were performed in serum-deprived methylcellulose cultures as described. The source of normal progenitor cells was narrow cell suspensions prepared from the tibia of DBA/2 mice. 3HTdR incorporation. The presence of hematopoietic growth factors in CM from erythroleukemia cell lines was routinely assayed on the basis of the capacity of the CM to stimulate 3HTdR incorporation by FDC-P1 and 32D cells. FDC-P1 is known to respond to both GM-CSF and IL-3, with a similar concentration/response curve. For both GM-CSF and IL-3, 3HTdR incorporation becomes significantly different from background at a growth factor concentration of 0.5 U/mL and the 3HTdR response plateaus at 10 U/mL. In contrast, 32D cells respond to low concentrations of IL-3 (1 U/mL) while the response to GM-CSF starts at a higher concentration (50 U/mL) and unpublished observations, July 1990). For the 3HTdR incorporation assay, FDC-P1 or 32D cells (10⁶ cells per 100 µL), after extensive washing in fresh medium not containing growth factors, were cultured in 96-well microtiter plates containing IMDM supplemented with 1% (wt/vol) bovine serum albumin (BSA). Twenty hours later, 3HTdR (0.25 µCi/well) was added to the wells and the incubation was continued for another 4 hours. Cells were subsequently harvested and adsorbed onto glass fiber filters and the 3HTdR counted in a Packard (Meriden, CT) liquid scintillation counter in the presence of Enhancer.

Southern blot analysis. High molecular weight genomic DNA was prepared by the procedure of Herrmann and Frischhaut. The spleens of noninfected NFS, BALB/c, or BALB/c × DBA/2 F1 mice (Friedrich Cancer Research and Development Center, Frederick, MD) were used as control. The DNA (20 µg) was digested with various restriction enzymes, separated by electrophoresis on 0.8% agarose gel, and transferred to a nylon membrane (Zeta-Probe; Bio-Rad, Richmond, CA). Polymerase chain reaction (PCR) products were separated on a 1.5% agarose gel. Membranes were baked at 80°C for 30 minutes in a vacuum oven and hybridized with cDNA or oligonucleotide probes as described below.

cDNA probes. Membranes were prehybridized at 43°C for 4 hours in a solution containing 50% formamide, 0.25 mol/L NaH₂PO₄ (pH 7.2), 0.25 mol/L NaCl, 10 mmol/L EDTA (pH 8.0), and 100 µg/mL heat-denatured salmon sperm DNA. Hybridization was performed in the same solution plus 32P-labeled probe at 43°C for 20 hours. After three sequential washings (2X SSC, 0.1% sodium dodecyl sulfate [SDS] and 0.5X SSC, 0.1% SDS, both at room temperature, and in 0.1X SSC, 0.1% SDS at 65°C), the membranes were autoradiographed with Kodak XAR-5 film (Eastman Kodak, Rochester, NY) and intensifying screens at ~70°C. The probes were 32P-labeled by the random nucleotide priming method (Amersham International, Amersham, UK). The probes used in this study were the HindIII-NcoI fragment of the murine IL-3 cDNA clone, the BamHI-EcoRI fragment of the murine GM-CSF cDNA clone, the 1.2-kb EcoRI fragment of the murine PU.1 cDNA clone, and the PstI fragment of the bovine β-actin cDNA clone.

Oligonucleotide probe. Membranes were prehybridized at 50°C overnight in a solution containing 5X SSC, 20 mmol/L NaH₂PO₄ (pH 7.0), 7% SDS, 10X Denhardt’s, and 100 µg/mL heat-denatured salmon sperm DNA. Hybridization was performed in the same solution plus 32P-labeled probe at 50°C overnight. The membranes were then washed twice in 3X SSC, 10X Denhardt’s, 5% SDS, 25 mmol/L NaH₂PO₄ (pH 7.5) at 50°C for 30 minutes and then in 1X SSC, 1% SDS at 50°C for 30 minutes. The GM-CSF gene-specific oligonucleotide probe 5'-GGCTTCTTTGATGCGCCTTCACTATGCTTCAAGGGCAG-3' was labeled by phosphor-
ylation with bacteriophage T4 polynucleotide kinase (New England Biolabs, Beverly, MA).

Northern blot analysis. Total RNA was prepared from cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method. Thirty micrograms of total RNA were denatured with 6% formaldehyde, fractionated by electrophoresis on a 1.2% agarose gel, and blotted on nylon membranes. Hybridization was performed with the same procedure described for Southern blot analysis.

RNA detection by PCR. One microgram of total RNA was used to generate first-strand cDNA using an oligo (dT) 12-18 primer and M-MLV reverse transcriptase ( Gibco BRL, Grand Island, NY). Amplification was performed with Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) and IL-3- or GM-CSF-specific primers (Ambion, CLONTECH Laboratories, Palo Alto, CA) according to the supplier's instructions. Thirty cycles consisting of denaturation at 94°C for 1 minute, annealing at 60°C for 2 minutes, and polymerization at 72°C for 3 minutes were performed in a Perkin-Elmer Cetus DNA Thermal Cycler. The product was analyzed by Southern blot using an IL-3 cDNA probe (HindIII-XbaI fragment) or the GM-CSF-specific oligonucleotide probe described above.

RESULTS

Effects of CM from SFFVp-induced erythroleukemia cell lines on the proliferation of normal progenitors or growth factor-dependent cell lines. Five erythroleukemia cell lines were used in this study. Four cell lines (MEL NP1, NP5, NP12, and NP13) were obtained from mice infected with helper-free SFFVp, and one cell line (DS19) was obtained from a mouse infected with an F-MuLV pseudotype of SFFVp. In preliminary experiments we showed that CM from some of the erythroleukemia cell lines analyzed contained an activity that stimulated GM colony formation by normal progenitor cells (Table 1), by FDC-P1 cells (Fig 1), or by 32D cells (not shown). The stimulatory effects of these CM on the proliferation of growth factor-dependent cell lines were analyzed in detail by the 3HTdR incorporation assay. As shown in Fig 2, all CM from the erythroleukemia cell lines examined stimulated the proliferation of 32D and/or FDC-P1. CM of four of the five lines investigated (MEL NP5, NP12, NP13, and DS19) induced 3HTdR incorporation of both 32D and FDC-P1 cells, whereas CM from MEL NP1 contained an activity that induced proliferation mainly of FDC-P1 cells.

An IL-3–specific MoAb (Fig 2) neutralized the effects of CM from four of the lines (MEL NP5, NP12, NP13, and DS19) on 3HTdR incorporation of 32D cells and a GM-CSF–specific antisem partially neutralized (≈30%) the effects of CM from three of the lines (MEL NP1, NP13, and DS19) on 3HTdR incorporation into FDC-P1 cells.

Table 1. Effect of CM From MEL NP12 or NP13 Cell Lines on the Proliferation of Normal Murine Progenitors in Semisolid Culture

<table>
<thead>
<tr>
<th>Source of Conditioned Medium</th>
<th>GM Colonies/5 x 10⁵ Marrow Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEL NP12 CM (20%)</td>
<td>129</td>
</tr>
<tr>
<td>MEL NP13 CM (20%)</td>
<td>71</td>
</tr>
<tr>
<td>MEL NP1 CM (20%)</td>
<td>5</td>
</tr>
<tr>
<td>MEL NP5 CM (20%)</td>
<td>120</td>
</tr>
<tr>
<td>MEL NP12 CM (10%)</td>
<td>2</td>
</tr>
<tr>
<td>MEL NP13 CM (10%)</td>
<td>22</td>
</tr>
<tr>
<td>MEL NP12 CM (5%)</td>
<td>12</td>
</tr>
<tr>
<td>MEL NP13 CM (5%)</td>
<td>10</td>
</tr>
<tr>
<td>MEL NP12 CM (2%)</td>
<td>9</td>
</tr>
<tr>
<td>MEL NP13 CM (2%)</td>
<td>5</td>
</tr>
<tr>
<td>MEL NP12 CM (1%)</td>
<td>5</td>
</tr>
<tr>
<td>MEL NP13 CM (1%)</td>
<td>1</td>
</tr>
</tbody>
</table>

*In this experiment CM was added to the culture without previous concentration.

To confirm that the ability of the SFFVp-induced cell lines to produce growth factors was associated with leukemic transformation and not simply acquired in more than 3 years of passage in vitro, we measured the stimulatory activity released by early passages (3 weeks after the establishment of the lines) of three of these cell lines. As shown in Table 2, CM from early passages stimulated the

Fig 1. Effect of CM from F-SFFVp–induced cell lines on the cloning of FDC-P1 cells in serum-deprived cultures. The number of colonies obtained in the presence of GM-CSF (2 U/mL) or IL-3 (1 U/mL) is presented for comparison. The CM were obtained after 2 days' incubation under serum-deprived conditions and were tested at a final concentration of 20% (vol/vol). Data represent the mean of one experiment performed in duplicate. The variability between the two values observed for each experimental point was ±10%.

Fig 2. Effect of CM from F-SFFVp–induced cell lines on 3HTdR incorporation by FDC-P1 (left) or 32D (right) cells. The incorporation of 3HTdR by FDC-P1 and 32D cells was performed in the presence (□) or in the absence (□) of neutralizing antibodies specific for GM-CSF (left panel), 1,500 final dilution, and IL-3 (right panel, 1,400 final dilution). All CM were concentrated 20-fold and added to the assay at a final concentration of 25% (vol/vol). IL-3 (1 U/mL) and low (2 U/mL) or high (50 U/mL) concentrations of GM-CSF were used as positive controls. Data represent the mean (±SD) of one experiment performed in triplicate. Similar data were obtained in two additional experiments.
In the absence of irradiated cells, the levels of expression of the IL-3 and GM-CSF genes by the erythroleukemia cell lines investigated were below the limit of detection of Northern analysis of either total or poly A-selected RNA (data not shown). For this reason, we used PCR to amplify IL-3 and GM-CSF transcripts. An IL-3-specific DNA fragment of the expected size (490 bp) was detected in four of five erythroleukemia cell lines investigated (MEL NP5, NP12, NP13, and DS19) as well as in concanavalin A (ConA)-stimulated spleen cells (Fig 5A). Shorter fragments (390, 270, and 90 bp) were also detected in the cell lines. We believe that these fragments are not caused by gene rearrangement but represent artifacts intrinsic to PCR amplification of nonabundant mRNA species. In fact, these fragments were also present in mRNA amplified from ConA-stimulated spleen cells, although at lower ratios (data not shown). As shown in Fig 5B, GM-CSF–specific DNA fragments were also detected in the same four erythroleukemia cell lines (MEL NP5, NP12, NP13, and DS19).

Southern blot analysis of the IL-3 and GM-CSF genes. To investigate whether the activation of the IL-3 or GM-CSF...
genes in the Friend cell lines was associated with gene rearrangements, we performed Southern blot analysis of the genomic DNA obtained from these lines. No gene rearrangement was detectable within the following IL-3 and GM-CSF regions: IL-3—7.8 kb of 5' flanking region, coding region, and 7.4 kb of 3' flanking region; GM-CSF—3.5 kb of 5' flanking region, coding region, and 3.3 kb of 3' flanking region (results not shown). These findings exclude the possibility that a proviral genome larger than approximately 200 bp (IL-3 and GM-CSF coding regions) or approximately 500 bp (IL-3 and GM-CSF flanking regions) was integrated in the IL-3 or GM-CSF gene loci.

Growth factor production by primary erythroleukemia cells induced by Friend SFFVp. To determine the correlation, if any, between activation of the expression of the IL-3 and GM-CSF genes and progression of SFFVp-induced erythroleukemia, we prepared CM from SFFVp-infected hyperplastic or leukemic spleen cells and analyzed the capacity of the CM to stimulate 3HTdR incorporation by 32D cells. CM from mock-infected spleen cells was analyzed for comparison.

CM from mock-infected spleen cells stimulated the 3HTdR incorporation of 32D cells (Table 4). However, this background activity is only 1% of the activity released by ConA-stimulated spleen cells (results not shown) and may be caused by the indirect effects on T cells of macrophages activated by adherence to plastic during the preparation of CM.33 Two to 3 weeks post-SFFVp infection, a 13-fold increase in spleen weight was observed. Erythroblasts represented more than 99% of the cell populations in these spleens. On the basis of the capacity of the splenic erythroblasts to generate cell lines in culture, we divided the
spleens into two groups: hyperplastic and leukemic. Hyperplastic spleen cells did not generate cell lines and failed to stimulate \(^{3}\)HThdR uptake by 32D or FDC-P1 cells above background (Table 4). These results are in agreement with previous data which indicated that erythroblasts do not produce IL-3.\(^{34}\) In contrast, leukemic spleen cells proliferated in vitro and produced detectable levels of stimulatory activity (Table 4) that were, at least in part (30% to 40%), neutralized by anti-IL-3 or anti-GM-CSF antibody (results not shown).

Spi-1 expression in SFFVp-induced cell lines and leukemic spleen cells. In 95% of Friend-SFFVp-induced erythroleukemia cell lines, SFFV is found inserted near the Spi-1 gene.\(^{10,11}\) This results in lineage-inappropriate expression of this gene. The five SFFVp-induced erythroleukemia cell lines used in this study were examined for expression of the Spi-1 gene and all were shown to express Spi-1 mRNA at high levels (Fig 6A). Erythroleukemia cell lines induced by F-MuLV (IW4-1\(^{35}\) and TP3\(^{36}\)) did not express Spi-1 mRNA, consistent with previous reports. As expected,\(^{13}\) Spi-1 mRNA was found in mock-infected spleen cells, which contain significant proportions of monocytes and B cells (Fig 6B, lanes 1 and 4). The level of Spi-1 expression did not increase in ConA-stimulated spleen cells (Fig 6B, lane 7). In SFFVp-infected hyperplastic spleen cells, Spi-1 expression was undetectable (Fig 6B, lanes 5 and 6). In contrast, in SFFVp-infected leukemic spleens, Spi-1 expression was five to six times higher than that observed in mock-transfected cells (Fig 6B, lanes 2 and 3).

### DISCUSSION

Autocrine growth factor production can be an important step in tumor progression.\(^{37,38}\) In the case of virus-induced transformation, activation of growth factor production by retrovirus insertion has been documented in the spontaneous transformation of the factor-dependent cell line D35 in vitro\(^{19}\) and FDC-P1 in vivo,\(^{15}\) as well as in the case of WEHI-3, whose ability to produce IL-3 is due to retroviral insertion near the IL-3 gene.\(^{39}\) In the case of other leukemia viruses, such as Abelson MuLV, tumor progression is also correlated with the ability of the cells to grow in the absence of a feeder layer\(^{40}\) or IL-3.\(^{41}\) In this case, the cells acquire the ability to produce IL-3 or other growth factors,\(^{42}\) although the mechanism of growth factor gene activation is not linked to viral integration in the proximity of the genes.

The erythroleukemia induced by the Friend SFFV is a multistage disease. The first stage consists of a polyclonal proliferation of erythroblasts that have a limited self-renewal capacity and are unable to grow as established cell lines in vitro or when transplanted into syngeneic mice. Another stage of the disease begins 3 to 4 weeks later and consists of a monoclonal proliferation of malignant erythroblasts that now have an unlimited self-renewal capacity and have acquired the ability to grow as subcutaneous tumors in vivo and as continuous erythroleukemia cell lines.

In this study, we analyzed the relationship between the progression of the disease and the capacity of the infected cells to produce hematopoietic growth factors. The data are summarized in Table 5.

Our data indicate that activation of growth factor production is a common event during SFFV-induced disease progression. The CM of all of the virus-induced erythroleukemia cell lines examined in this study contained activities that stimulated the growth of normal progenitors as well as factor-dependent cell lines 32D or FDC-P1.

| Table 4. Effects of CM From SFFVp-Infected Splenens on \(^{3}H\)ThdR-Incorporation by 32D Cells |
|---------------------------|--------------------------------|
| Stimulus                  | Spleen Weight (g) | \(^{3}H\)ThdR-Incorporation (cpm \times 10^4) 32D |
| None                      | —                | 0.6 |
| IL-3 (20 U/mL)            | —                | 63  |
| GM-CSF (2 U/mL)           | —                | 13  |
| GM-CSF (50 U/mL)          | —                | 31  |
| SFFVp-infected hyperplastic spleens (14 spleens) | 0.09 ± 0.02 | 6.6 ± 1.8 |
| SFFVp-infected leukemic spleens (7 spleens) | 1.85 ± 0.7 | 0.5 ± 0.7 |
| Mock-infected spleens (7 spleens) | 1.19 ± 0.5 | 7.6 ± 0.7 |

Fig 6. Spi-1 expression in F-SFFVp-induced erythroleukemia cell lines (A) and in spleen cells of mice infected with F-SFFVp. (A) F-MuLV–induced cell lines (IW4-1 and TP3) were used as negative control for Spi-1 expression. (B) Mock-infected (lanes 1 and 4) and ConA-stimulated (lane 7) spleen cells had detectable levels of Spi-1 mRNA. With F-SFFVp infection, leukemic (lanes 2 and 3) but not hyperplastic (lanes 5 and 6) spleen cells showed detectable levels of Spi-1 mRNA. MEL NP12 cells served as a positive control. The RNA was prepared from the same spleens used to prepare CM and presented in Table 4. The membrane was stripped and rehybridized with a probe specific for \(\beta\)-actin to normalize the amount of RNA loaded.
Viral integration in or near the Spi-1/PU.1 site and the grow in culture, produce IL-3 and/or GM-CSF (Table because spleen cells from some of the leukemic mice and one of the SFFVp-induced cell lines (MEL NP1) did not grow as cell lines in culture, do not produce IL-3 or GM-CSF (Table 4). However, expression of Spi-1 does not always correlate with IL-3 or GM-CSF production because spleen cells from some of the leukemic mice and one of the SFFVp-induced cell lines (MEL NP1) did not produce either of these growth factors, despite the fact that they all expressed Spi-1. Attempts to inhibit growth factor production in SFFVp-induced cell lines with antisense oligonucleotides specific for the Spi-1/PU.1 gene were unsuccessful because of the high levels of toxicity exerted by the oligonucleotides in our assay. Therefore, we cannot firmly conclude that Spi-1 activation induced the production of IL-3 and GM-CSF in the SFFVp-infected cells.

Our results suggest that growth factor production may play a role in immortalizing SFFV-infected erythroblasts, resulting in their increased self renewal and ability to grow outside of their normal microenvironment. Although the cell lines studied here can grow in serum-containing medium without added growth factors, their growth under serum-free conditions, like that of Rauscher erythroleukemia cells, depends on a high cell density or the addition of an irradiated feeder layer. Interestingly, the stringency for the feeder-layer requirement was inversely correlated with the level of growth factors released by the cells (MEL NP13 > MEL NP12 > DS19 = MEL NP5 > MEL NP1). This suggests that the cells are, indeed, dependent on some factor(s) that can be produced by the cells themselves. However, it is not yet known if IL-3 and GM-CSF are autocrine factors for these cells. We are currently analyzing the SFFV-induced erythroleukemia cell lines for receptors for IL-3 and GM-CSF to determine whether IL-3 or GM-CSF production by these cells results in their autocrine growth.

NOTE ADDED IN PROOF

After this report was accepted for publication, we were provided with the murine IL-3 receptor (R) probe (AIC2A) to analyze the expression of the IL-3R genes in several factor-dependent cell lines by S1 nuclease protection analysis. Figure 7 shows the results of one such experiment in which mRNA from MEL NP12 was analyzed. MEL NP12 protects from S1 digestion the radiolabeled AIC2A probe generating fragments indistinguishable from those obtained with the 32D cell lines and of the sizes expected for the α (AIC2A) and β (AIC2B) subunits of the murine IL-3R. These data strongly support the hypothesis that the interaction between IL-3 and its receptor contribute, at least in part, to promote proliferation of the MEL NP12 cell line.

Table 5. Summary of the Data

<table>
<thead>
<tr>
<th>Cell Line (ref)</th>
<th>Target Cells and Stimulatory Activity of Cell Line CM*</th>
<th>mRNA Present in Cell Lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mouse Strain</td>
<td>32D</td>
</tr>
<tr>
<td>MEL NP1 (8)</td>
<td>SFFVp</td>
<td>BALB/c</td>
</tr>
<tr>
<td>MEL NP5 (8)</td>
<td>SFFVp</td>
<td>BALB/c</td>
</tr>
<tr>
<td>MEL NP12 (8)</td>
<td>SFFVp</td>
<td>NFS</td>
</tr>
<tr>
<td>MEL NP13 (8)</td>
<td>SFFVp</td>
<td>NFS</td>
</tr>
<tr>
<td>DS19 (18)</td>
<td>F-MuLV + SFFVp</td>
<td>DBA/2</td>
</tr>
<tr>
<td>Hyperplastic spleen cells</td>
<td>F-MuLV + SFFVp</td>
<td>DBA/2</td>
</tr>
<tr>
<td>Leukemic spleen cells</td>
<td>F-MuLV + SFFVp</td>
<td>BALB/c</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

*The stimulatory activity was measured by [H]Tdr assay and was neutralized either by an IL-3-neutralizing monoclonal (32D) or anti-GM-CSF antiserum (FDC-P1).

In four of five cases, we were able to demonstrate the production by these cell lines of GM-CSF and/or IL-3. This demonstration was based on the spectrum of biologic activity released by the cells, on the biochemical and immunologic characteristics of the colony-stimulating activity, as well as on the presence of specific mRNA transcripts. When the CM from one of these lines (MEL NP12) was fractionated, we could also detect growth-promoting activity that could not be neutralized by either the IL-3- or GM-CSF-specific antibodies. This activity might represent another growth factor, similar to the one produced by Rauscher cells or stem cell factor.

Unlike the results obtained during the transformation of the factor-dependent cell line D35 in vitro or FDC-P1 in vivo, it does not appear that proviral integration in the proximity of the IL-3 or GM-CSF genes in the erythroleukemia cell lines examined here is responsible for their activation. No gene rearrangement was detectable at the GM-CSF or IL-3 loci using Southern blot analysis.

The growth factor production by these cells may be due to an indirect effect of SFFV integration. All of the cell lines analyzed in this study express Spi-1 (Fig 6A), which is a putative oncogene cloned from erythroleukemic cells as a transcribed region directly adjacent to the integration site of SFFV. Spi-1 is known to be identical to PU.1, a tissue-specific transcription factor whose cognate sequence is represented by the PU box (a purine-rich sequence 5'-GAGGAA-3'), which is also present in the promoter region of the GM-CSF and IL-3 genes. Therefore, it is possible that activation of IL-3 or GM-CSF in SFFV-induced erythroleukemia cell lines is a consequence of the viral integration in or near the Spi-1/PU.1 site and the production of this transcription factor in an erythroid environment. In support of this is our observation that hyperplastic SFFVp-infected erythroblasts, which do not grow as cell lines in culture, do not produce IL-3 or GM-CSF (Table 4) and do not express Spi-1 (Fig 6B). In contrast, leukemic SFFVp-infected erythroblasts, which grow in culture, produce IL-3 and/or GM-CSF (Table 4) and express Spi-1 (Fig 6B). However, expression of Spi-1 does not always correlate with IL-3 or GM-CSF production because spleen cells from some of the leukemic mice and one of the SFFVp-induced cell lines (MEL NP1) did not grow in culture, do not produce IL-3 or GM-CSF (Table 4). However, expression of Spi-1 does not always correlate with IL-3 or GM-CSF production because spleen cells from some of the leukemic mice and one of the SFFVp-induced cell lines (MEL NP1) did not...
Fig 7. S1 nuclease protection analysis of the IL-3R in 32D and MEL NP12 cell lines (25 μg of RNA in both cases). The S1 nuclease protection analysis was performed using the AIC2A probe (kindly provided by Dr Atsushi Miyajima, DNAX, Palo Alto, CA) as described. Negative controls were represented by tRNA and mRNA from a human IL-3-dependent cell line, UT-7 (the mouse IL-3R probe does not crosshybridize with the human IL-3R). The expected bands for the α (AIC2A) and β (AIC2B) subunits of the IL-3R were detected in both 32D and MEL NP12 cell lines as indicated on the right. Size markers (6X174-HaeIII) (in bp) are indicated on the left.

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REFERENCES


Expression of the interleukin-3 and granulocyte-macrophage colony-stimulating factor genes in Friend spleen focus-forming virus-induced erythroleukemia

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